Interfacial action of phospholipase A2

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Chapter 1

Introduction

1.1 Introduction

1.1.1 History, lipases, phospholipases and phospholipase A_2

Phospholipase A_2 (PLA_2) is a relatively small enzyme that hydrolyses the sn-2 ester bond of phospholipids. The discovery of PLA_2 was based on the observation that pancreatic juice and cobra venom were able to hydrolyze phosphatidyl choline.[1] Phospholipases A_2 are widespread in nature,[2] and occur in many human and animal tissues, both intra- and extracellularly. Intracellular phospholipases A_2 are likely to be the main mediators of inflammatory response. The action of the intracellular phospholipases produces arachidonic acid, which is a mediator of an inflammatory response cascade. The extracellular phospholipases A_2 have a digestive function, and can be found in large amounts in mammalian pancreatic juice and in snake and bee venoms. The phospholipase A_2 discussed in this thesis is porcine pancreatic PLA_2.

1.1.2 Nomenclature

The phospholipases can be divided into groups depending on which glycerol ester bond they are capable of cleaving.[3] These bonds are marked in figure 1.1. The phospholipases are thus called phospholipase A, B, C, and D. For the phospholipases A, a subscript 1 or 2 is added depending on whether the cleaved bond involved is at the sn-1 or sn-2, as in our case, position of the phospholipid substrate.

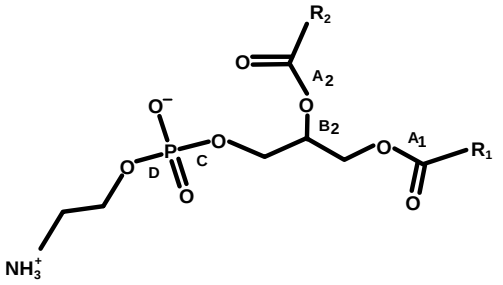

Figure 1.1: Site of action of the phospholipases. A_1, A_2, B_2, C and D mark the position where these phospholipases break a bond of the phospholipid during catalysis.

1.2 Structure

Crystal structures of several extracellular phospholipases A_2 are available from many different organisms and tissues, e.g. bovine pancreatic,[5–9] porcine pancreatic,[10,11] bee venom,[12] snake venom,[13–16] and human platelet.[17,18] The most interesting fact of all these structures is that the structural difference between this variety of phospholipases A_2 is very small, and the sequences show a great deal of homology.

The X-ray structure of porcine pancreatic phospholipase A_2 has been determined by Dijkstra[10] and Finzel.[11] The primary sequence of PLA_2 is displayed in figure 1.4 and a ribbon presentation is given in figure 1.2. The total mass of this 124 residue protein is 14 kilodalton. 50% of the residues of PLA_2 have an α-helical conformation forming five helices. A small anti-parallel β-sheet is located next to helix A, the N-terminal α-helix.

The active site of the PLA_2 is connected to the protein surface via a hydrophobic channel, the hydrophobic cleft. This cleft is constructed from mainly hydrophobic residues. Phospholipid substrates move through the hydrophobic cleft towards the active site of the protein. A heptacoordinated calcium ion is present in the active site. This calcium ion is essential for activity, and is ligated by three carbonyl backbone oxygen atoms (residues Tyr28, Gly30, and Gly32), two carboxyl oxygen atoms (the sidechain of Asp49), and two water molecules. The region around the calcium ion that forms its binding pocket (see figure 1.2 and figure 2.1 on page 24) is formed by residues 25 to 42 and is called the calcium binding loop.[6,19]

An important structural factor in PLA_2 is the hydrogen-bonded network. A schematic presentation of the N-terminal hydrogen-bonded network as it is found in the crystal structure of PLA_2[6] is given in figure 1.3. This network is not expected

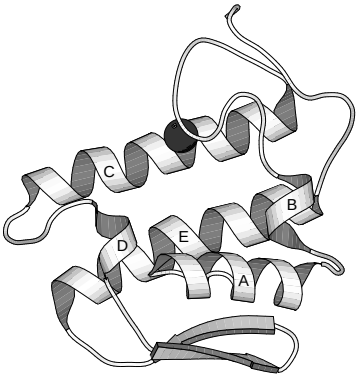

Figure 1.2: Ribbon presentation of the crystal structure of PLA_2. The five α-helices are marked with their identifier. The anti-parallel β-sheet is located next to helix A at the bottom of this picture. The dark sphere on top of helix C represents the calcium ion that is bound to the protein via the calcium binding loop.

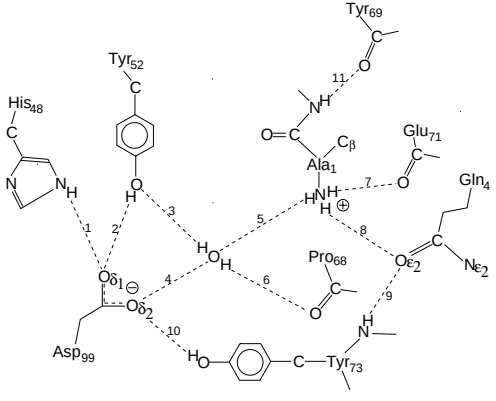

Figure 1.3: Schematic presentation of the hydrogen bonded network as it is found in the crystal structure of porcine pancreatic PLA_2. The hydrogen bonded network connects the N-terminus (Ala1) to the active site. The hydrogen bonds (1,...,11) are represented by the dashed lines.

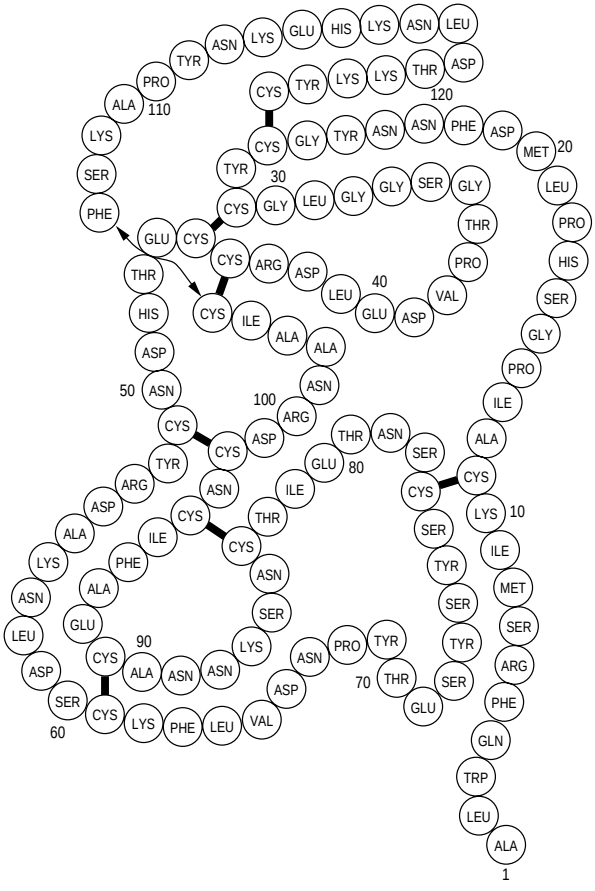

Figure 1.4: Primary amino acid sequence of porcine pancreatic PLA_2. The connections of the seven disulfide bridges are indicated (from Fleer[4]).

to be essential for catalytic activity.[20,21] It links the residues of the catalytic triad (formed by residues Asp99, His48 and a water molecule) to the N-terminus.

1.3 Catalysis mechanism

The catalysis mechanism of the chemical reaction of PLA_2 was proposed by Verheij[22] in 1980. This mechanism, graphically displayed in figure 1.5 is analogous to that of the serine proteases. In PLA_2 the serine residue of the catalytic Asp-His-Ser triad of the serine proteases is replaced by a water molecule. The calcium ion, to which the phospholipid substrate is bound, orients and polarizes the carbonyl group of the phospholipid, which results in a more positive charge on this carbonyl carbon atom. This is in favor of the nucleophilic attack by the catalytic water molecule.

1.4 Kinetics

The activity of PLA_2 is strongly enhanced by aggregated substrates like micelles or mono-layers. This was already reported by Pieterson et al in 1974.[24] When the substrate concentration reaches the Critical Micelle Concentration (CMC), the enzyme activity is enhanced by several orders of magnitude (see figure 1.6). This process, where the protein is activated by interaction with a phospholipid interface, is called interfacial activation.[24] Several models have been suggested to explain this phenomenon.[25]

The *Substrate models*[15,26,27] attribute the enhanced activity of the protein to modified properties of the substrate, caused by the aggregation process. The most important features of these models are the higher substrate concentration caused by the aggregation of the substrate, a favorable conformation of phospholipid molecules in the interface, dehydration of the sn-2 ester-bond and facilitated diffusion of reaction products. A central theme in these models is that the protein is thought to be structurally invariant, irrespective of the absence or presence of a phospholipid-water interface. The conformation of the protein will not show a change upon binding to the interface.

The *Enzyme models*[25,28–30] view the enhanced activity of the protein as regulated by the phospholipid-water interface. The main difference with the substrate models is that the enzyme is thought to undergo a conformational change, caused by interaction with the phospholipid-water interface. The Interface Recognition Site (IRS)[30] model is a very important enzyme model. In this model the enzyme binds the phospholipid-water interface via a special site located on one face of the protein. The binding of this site to the interface results in a conformation change of the protein, and the protein changes from an inactive to a catalytically active state.

Several enzymes, members of the family of the lipases, show a remarkable conformation change upon binding aggregated substrate.[31–33] The conformation change is

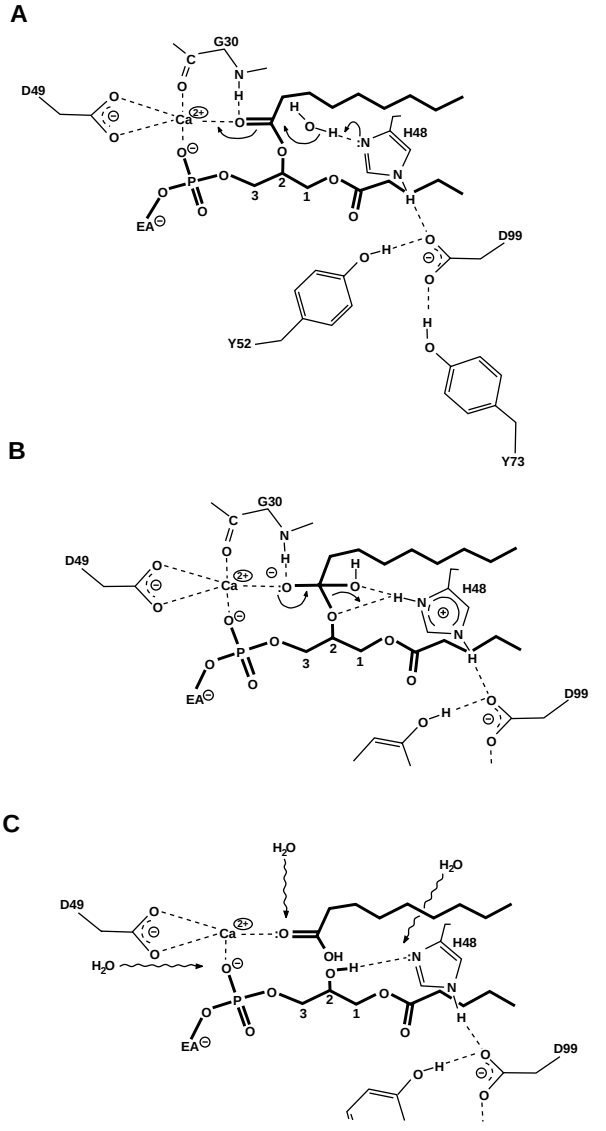

*Figure 1.5: Schematic representation of the catalytic mechanism of PLA_2 as proposed by Verheij et al. in 1980[22] (from van den Berg[23]) . The catalysis starts with the transfer of a proton of the catalytic water molecule to His48. The hydroxyl group binds to the polarized carbonyl carbon atom of the phospholipid (**A**). In the next step, the proton on His48 is transfered back to the phospholipid ester oxygen atom (**B**). This results in a breakage of the ester bond (**C**).*

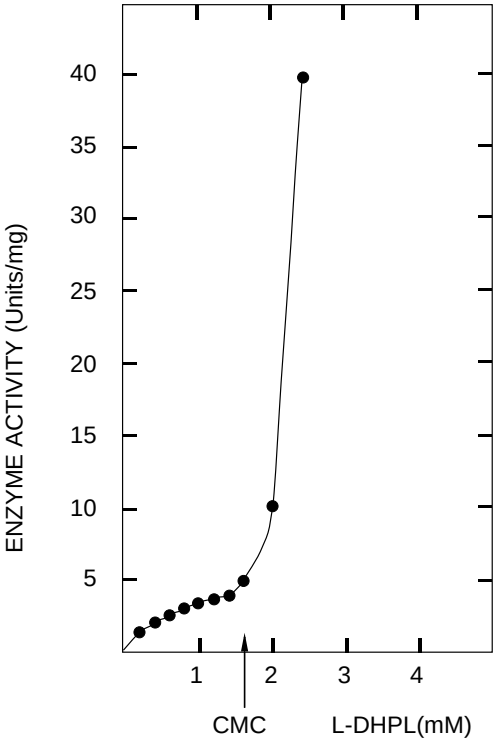

Figure 1.6: The activity of porcine pancreatic PLA_2 as a function of phospholipid (in this case L-diheptanoyllecithin) concentration. The "jump" in activity upon nearing and passing the Critical Micelle Concentration (CMC) is called "interfacial activation" (from Pieterson et al.[24]).

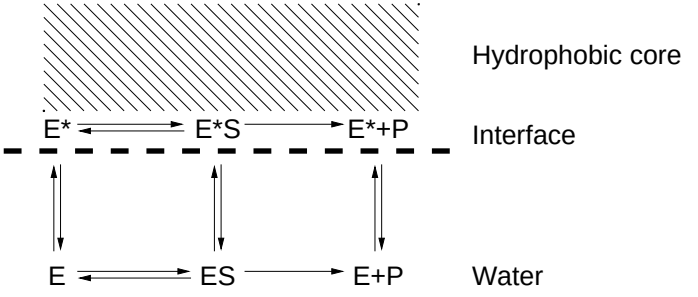

Figure 1.7: Schematic representation of the action of PLA_2 at a phospholipid-water interface, according to the Interfacial Recognition Site (IRS) theory. E^* represents the "activated" form of the enzyme which is formed upon the interaction of the enzyme with the phospholipid-water interface.[25]

the movement of a flap which results in exposure of the catalytic site to the phospholipid monolayer. In contrast to these lipases, PLA_2 does not change its structure upon membrane or micelle binding. Crystal structures of native, and inhibited enzymes have the same backbone structure,[13,18,34] and thus support the substrate models. NMR-experiments, on the other hand, show a more distinct conformational change between native and activated enzyme.[23,35,36] The main conformation changes are located in the N-terminal residues and in a surface binding loop (residues 62-72). The residues in this surface loop have a more disordered conformation in the native state compared with the active state. In activated PLA_2 the N-terminal residues are completely α-helical (helix A) while these residues are disordered in solution, i.e. the native state. Moreover, crystallographic[7,18] studies have shown that there are some small conformational changes which take place when an inhibitor is bound to the active site of PLA_2. The main conformation change is located in the N-terminal helix which shows a small shift towards the calcium ion of PLA_2.

1.4.1 Quantitative aspects

The action of phospholipase A_2 at interfaces has been studied extensively in various environments like monolayers,[37–40] micelles[41] and bilayer vesicles.[42–48] The kinetics of the action of the protein towards substrate aggregates can be analyzed using the classical Michaelis-Menten theory adopted for the action of enzymes at interfaces.[28] The action of phospholipase A_2 is plotted in figure 1.7. The Michaelis-Menten scheme for the interfacial catalysis reaction of PLA_2 is

$$\mathrm{E} \underset{K_d}{\overset{K_p}{\rightleftharpoons}} \mathrm{E}^* \,;\; \mathrm{E}^* + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}^*\mathrm{S} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E}^*\mathrm{P} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E}^* + \mathrm{P}$$

where E, S, and P represent enzyme substrate and product, respectively. The asterisk (*) marks that the species is located at the interface. In this reaction scheme, the pathways of inhibition are not shown. We note that concentrations of S or involving E*, are expressed in mol per unit membrane surface.

In this reaction scheme, the back reaction from E*P to E*S can be neglected because from experiment it is known that once the sn-2 ester bond of the phospholipid is hydrolyzed, the products are released and resynthesis of the original phospholipid does not occur.[43,49] This establishes that $k_3 \gg k_{-2}$. Thus k_{-2} maybe assumed to be 0.

The two reaction steps from E* to E* + P are often written as one step

$$\mathrm{E}^*\mathrm{S} \overset{k_{cat}}{\rightarrow} \mathrm{E}^* + \mathrm{P}$$

where k_{cat} is the effective catalytic rate constant, which is defined by k_2 and k_3.[45]

$$k_{cat} = \frac{k_2}{1 + \frac{k_2}{k_3}}$$

If the chemical reaction (E*S $\rightarrow$ E*P) is the rate determining, or slowest, step: $k_3 \gg k_2$ and $k_{cat} = k_2$. If on the other hand the product release step is the rate determining step: $k_3 \ll k_2$ and $k_{cat} = k_3$.

A theoretical description of the rate of product accumulation, for a protein present at the interface E*, can be derived by application of the steady state approximation.

$$\frac{d[\mathrm{P}]}{dt} = \frac{k_{cat}\,[\mathrm{E}^*][\mathrm{S}]}{K_M^* + [\mathrm{S}]}$$

Here K_M^* is the Interfacial Michaelis-Menten constant. The experimental determination of these catalytic rate constants is performed by changing the substrate concentration. As the properties of the interface, in which the substrate is located is not allowed to change, the concentration is varied by mixing the phospholipid substrate

with an inactive substrate analogue which does join the catalytic process.[50] It is difficult to incorporate such an inactive phospholipid (used as 'solvent') that does not influence the interface, or the interactions with the protein in any way. The value of k_{cat} in a 1,2-diacyl-*sn*-glycerol-3-phosphocholine (DDPC) micelle environment, reported by Slotboom et al. in 1976,[41] was determined at a value of 140 phospholipid molecules s^{-1} protein molecule^{-1}. Since the maximum rate of product accumulation V_{max} is proportional to k_{cat}, the maximum product accumulation rate per protein is 140 phospholipid molecules s^{-1}.

$$V_{max} = k_{cat}\,[\mathrm{E}^*]$$

When the product accumulation rate is measured in a Langmuir trough in the preseence of a monolayer environment at the air-water interface[51] at the same conditions as used in our simulations (see chapter 4), the product accumulation rate is at least 4 phospholipid molecules s^{-1} protein molecule^{-1}. This rate is the minimal rate as it is dependent on the concentration of E*. This concentration is approximated by measuring, using radioactive labeled PLA_2, the amount of protein present at the interface, and assuming that all of these proteins are active at the interface. The actual rate will be underestimated by this technique. We conclude that the product accumulation rate at a monolayer is between 4 and 140 lipid molecules per protein molecule, and probably closer to the latter.

1.5 Molecular Dynamics

Molecular Dynamics (MD) is the computational simulation method used in this thesis. The method of MD solves *Newton's* equations of motion for a molecular system which is usually a box filled with atoms, the particles.

$$m_i \frac{d^2 \boldsymbol{r}_i(t)}{dt^2} = \boldsymbol{F}_i(\boldsymbol{r}_1, \ldots, \boldsymbol{r}_N)$$

Here $\boldsymbol{r}_i$ are the position vectors of the particles with mass m_i and $\boldsymbol{F}_i$ is the force depending on the positions of all N particles in the system. The force $\boldsymbol{F}_i$ is obtained from the derivative of the interaction potential V between the particles.

$$\boldsymbol{F}_i = -\frac{\partial V(\boldsymbol{r}_1, \ldots, \boldsymbol{r}_N)}{\partial \boldsymbol{r}_i}$$

The interaction potential V contains in principle all the necessary information to describe the system properly and contains for example all the bond, angle, Lennard-Jones, and Coulomb interactions.[52]

Newton equations are solved stepwise in time, and each step results in a new configuration of the molecular system, a few femto seconds later in time. Today most simulations have a length of several nanoseconds, and thus require this calculation to be repeated at least 500,000 times. All the calculated configurations together form a trajectory which describes the motions and locations of all the particles during simulated time. From this trajectory a variety of properties, the behavior of the system, can be calculated. Using statistical mechanics, various statistic, dynamic and thermodynamic properties can be computed. Thus MD is in principle a method which calculates the macroscopic properties from microscopic behavior. Therefore the trajectory should be a representative sample of the available configuration space. This means that a trajectory should be long enough in order to acquire a representative sample of the available configuration space. As computer power is limited, the adequate sampling of configuration space is still a major issue.

Computer power has increased tremendously over the last decades and still seems to increase by a factor of 10 each 5-7 years.[52] As a result of this both size and time scale of the systems simulated with MD increased. The first MD simulation, reported by Alder and Wainwright in 1957,[53] contained 108 hard sphere particles and had a simple potential. Nowadays complex biological systems with a system size of several tens of thousands of particles are standard.[54] The first MD simulation of a protein in explicit water with a length of 1 microsecond has been reported recently.[55]

1.6 Aim of this thesis

The aim of this thesis is to investigate the action of phospholipase A_2 from a 'Molecular Dynamics' point of view. Using MD it is possible to study several dynamical processes of PLA_2 in atomic detail and thus MD can provide a more detailed insight than other methods like X-Ray and NMR. Furthermore the large amount of data of PLA_2 that is available makes a good comparison between simulation and experiment possible and thus can provide more insight into the reliability of MD simulations. This thesis addresses the following questions.

- Does PLA_2 change its conformation upon monolayer binding? Previous results have shown that PLA_2 changes its conformation upon binding aggregated substrate. We want to use MD simulation methods to characterize the conformation changes that take place when PLA_2 is simulated in the presence of a phospholipid monolayer.
- Is it possible to predict the binding conformation of a substrate to PLA_2? As substrate binding to the active site of a protein is very interesting from a

biotechnological point of view, an attempt is made to set up a simulation in such a way that the binding of a substrate molecule to the active site of PLA_2 can be predicted.

- Can we calculate catalytic rate constants of PLA_2 from simulation? The accurate estimation of enzymatic catalytic rate constants from simulation can have enormous implications. For example the inclusion of such calculations in pharmacological research could potentially diminish the need for real experiments. However present simulations are often not good at predicting accurate binding constants of protein substrate complexes. Our aim here is then to set up simulations in such a way that accurate calculations of these binding constants become feasible.

1.7 Outline

1.7.1 Chapter 2: Startup

Chapter 2 describes several computational methods which are needed for the MD simulation in the other chapters of this thesis. These methods are needed to keep the MD simulations reliable and not too time consuming. The first and the second method have to do with polarization. Polarization is not included in the MD forcefield which can lead to severe errors in MD simulations. The first method solves this problem by using a static method which assumes that the polarization is constant during the simulation. The next method treats the polarization problem by using a dynamic solution which includes a distance dependent polarization. The third method is a protein docking method. Since conventional MD has a limited time scale a brute force docking procedure is presented. This method is used to calculate the optimal orientation of PLA_2 with respect to a phospholipid monolayer. This optimal configuration is used as a starting point for full detail MD simulations.

1.7.2 Chapter 3: Monolayer binding

Chapter 3 describes two simulations of PLA_2. The first simulation is that of PLA_2 free in solution. The second simulation is that of PLA_2 in the presence of a phospholipid monolayer. The trajectories of these two simulations are extensively analysed, and produce more insight into the differences in behavior of PLA_2 under the influence of these environments.

1.7.3 Chapter 4: Substrate binding

Chapter 4 describes the binding of a substrate molecule to PLA_2 for the case where PLA_2 is attached to a phospholipid monolayer. The binding of the phospholipid substrate molecule is discussed both qualitatively and quantitatively. For the qualitative part the binding conformation of the phospholipid is analyzed. For the quantitative part the product accumulation rate is estimated from the derivative of the potential of mean force, which is calculated from several constrained simulations.

1.7.4 Chapter 5: Concluding remarks

In the final chapter of this thesis an overall conclusion of this thesis is presented together with a perspective view of the future.